

Efficacy of an experimentally inactivated *Streptococcus agalactiae* vaccine in Nile tilapia (*Oreochromis niloticus*) reared in Brazil

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Abstract

Tilapia aquaculture is one of the fastest-growing segments of fish production in Brazil. Nile tilapia (*Oreochromis niloticus*) is largely cultivated in the state of Paraná, where *Streptococcus agalactiae* is the cause of severe disease outbreaks. The objective of this paper was to evaluate an inactivated *S. agalactiae* vaccine in tilapia for the control of streptococcal disease outbreaks. Tilapia, weighing approximately 20 g each, were intraperitoneally (i.p.) inoculated with 0.1 mL of the vaccine at a dose of 2.0×10^8 colony-forming unit (CFU) mL⁻¹. One group of tilapia (treatment 1) received one vaccine dose, and the other group of tilapia (treatment 2) received two doses, with an interval of 21 days. The control group was i.p. inoculated with 0.1 mL tryptic soy broth fish⁻¹. Immunized and control tilapia were i.p. challenged with 0.1 mL of 3.0×10^7 CFU mL⁻¹ at 30 days post vaccination. The fish were monitored daily for disease signs and for mortality for 16 days post challenge. A statistically significant difference ($P = 0.0045$) was found between the mortality of treatments 1 and 2. The value of relative per cent of survival of 83.6% and 96.4%, respectively, indicate that this vaccine was efficient in Nile tilapia.

Keywords: *Streptococcus agalactiae*, vaccine, efficacy, tilapia, intensive rearing system

Introduction

World aquaculture has rapidly grown over the last 50 years. In the beginning of the 1950s, production was

of approximately 1 million tonnes and by 2004, it reached 59.4 million tonnes. From this total, 897 276 tonnes were of tilapia (*Oreochromis* spp.). According to data from Food And Agriculture Organization of the United Nations, State of world aquaculture (2006), Brazil was the seventh producer in 2004, with a production of 69 078 tonnes. In the state of Paraná, the total production in that year reached 16 558 tonnes, being 72% (11 921 tonnes) of tilapia (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis 2005).

Worldwide, among the bacterial diseases, septicæmia caused by *Streptococcus* spp. is the most severe disease problem in intensively raised tilapia (Suresh 1999). *Streptococcus iniae* has been isolated from > 27 species of fish (Agnew & Barnes 2007). Eldar, Bejerano and Bercovier (1994) isolated *Streptococcus difficile*, later determined to be *Streptococcus agalactiae* (Kawamura, Itoh, Mishima, Ohkusu, Kasai & Ezaki 2005). *Streptococcus agalactiae* (Group B) has been shown to cause significant morbidity and mortality among a variety of freshwater and saltwater fish species throughout the world (Evans, Klesius, Gilbert, Shoemaker, Al Sarawi, Landsberg, Duremdez, Al Marzouk & Al Zenki 2002). Group B *Streptococcus* has been reported from six countries on three continents: United States, Israel, Japan, Kuwait, Thailand and Brazil. Countries in which both *S. iniae* and *S. agalactiae* have been reported include the United States, Israel, Japan and Thailand (Evans, Klesius & Shoemaker 2006). In Brazil, Salvador, Müller, Leonhardt, Pretto-giordano, Dias, Freitas and Moreno

(2003), Salvador, Müller, Freitas, Leonhardt, Pretto-Giordano and Dias (2005) and Figueiredo, Carneiro, Faria and Costa (2006) isolated *S. agalactiae* from diseased tilapia.

Streptococcus agalactiae also causes septicaemic disease in tilapia, affecting organs such as the brain, kidney and gut, among others. Most common disease signs include anorexia, exophthalmia, ascites and erratic swimming (Plumb 1999; Evans *et al.* 2002; Salvador *et al.* 2005). The increase in tilapia production has occurred mainly in intensive breeding systems that are characterized by high stocking rates. The greater contact between fish, constant and inadequate handling practices, associated with nutritional failures, can alter the quality of water, leading to the incidence of infectious diseases (Plumb 1997; Martins 1998; Shoemaker, Evans & Klesius 2000). In an attempt to control these diseases, producers have used antibiotic and chemical products, many times in an indiscriminate way, damaging the environment and possibly affecting consumers' health (Klesius, Evans & Shoemaker 2004). In that sense, research on development and use of bacterial vaccines in reared fish have been intensified in the last 10 years (Gudding, Lillehaug & Evensen 1999; Huising, Guichelaar, Hoek, Verburg-vankemenade, Flik, Savelkoul & Rombout 2003).

Different types of streptococcal vaccines, with or without addition of extracellular products, were experimentally studied (Klesius *et al.* 2004). The majority of research has involved *S. iniae* (Eldar, Horovitz & Bercovier 1997; Klesius, Shoemaker & Evans 2000; Shoemaker, Vandenberg & Desormeaux 2006). Eldar, Shapiro, Bejerano and Bercovier (1995) investigated two types of vaccines: one with inactivated cells of *S. difficile*, and the other with a protein extract from *S. difficile*. Both types of vaccine were found to be experimentally protective in tilapia. Toranzo, Devesa, Romalde, Lamas, Riaza, Leiro and Barja (1995) studied an inactivated vaccine from *Enterococcus* spp. cells supplemented with a toxoid. Akhlaghi, Munday and Whittington (1996) evaluated an experimental vaccine with inactivated cells of *Streptococcus* spp. with incomplete Freund's adjuvant. These vaccines were also found to be experimentally protective against *Enterococcus* spp. and *Streptococcus* spp. respectively.

Evans, Klesius and Shoemaker (2004) showed that tilapia immunized with the modified inactivated *S. agalactiae* vaccine were experimentally protected against *S. agalactiae*. However, this study also showed a lack of cross-protection of an *S. iniae* vaccine for *S. agalactiae* challenge.

The aim of this work was to evaluate the efficacy of a vaccine composed of inactivated *S. agalactiae* cells isolated from naturally infected Nile tilapia (*Oreochromis niloticus*) cultured in Brazil.

Materials and methods

Fish

A total of 412 Nile tilapia (*O. niloticus*) were used, which were kindly supplied by the Pisciculture Station of Animal and Vegetal Biology Department from the State University of Londrina. Fish used in this experiment had an average weight of 20 g, and were kept in fibreglass containers (500 L) with a water volume of 400 L and a renovation rate of 3 L water min⁻¹. Tilapia were placed in the fibreglass containers 10 days before the beginning of the experiment so that they could be acclimatized. Before beginning the experiment, three fish from each treatment were randomly necropsied. Tissues from the cranial kidney and brain were collected for bacteriological examination, with the objective of verifying whether the fish were free from *S. agalactiae*. Fish were fed with an extruded ration (30% gross protein, FishTM, Cooperativa Integrada, Londrina, Paraná, Brazil) at the rate of 3% live weight, twice a day.

Water quality control

The containers had semi-artisan well water, with a continuous flow and aeration, and cleaning was performed daily by suction. During the experiment, the average concentration of oxygen dissolved in the water was maintained at 3.8 ± 0.5 mg L⁻¹, temperature at 26.0 ± 0.8 °C, ammonia at 0.05 mg L⁻¹ and nitrites at 0.25 mg L⁻¹. The dissolved oxygen and the temperature were measured daily using equipment model YSI 55 (Yellow Spring Instrument, Yellow Springs, OH, USA). Ammonia was measured quarterly using the Berthelot reaction (Solaranzo 1969) and nitrite using the Griess reaction (Aminot & Chaussepied 1983).

Vaccine

Vaccine was prepared according to Klesius, Shoemaker and Evans (1999) and Evans *et al.* (2004), with some modifications. *Streptococcus agalactiae* (UEL 12), isolated from tilapia brain, of diseased fish cultivated in net-tanks in the northern part of the state of Para-

ná was used as the master seed. This tilapia exhibited clinical signs that included exophthalmia, ascites and erratic swimming. The vaccine was fermented in 1000 mL of tryptic soy broth (TSB Difco Laboratories, Sparks, MD, USA) at 30 °C for 72 h from a thawed aliquot of the master seed. In order to verify the purity of the culture, a single colony was streaked on Columbia agar (Difco Laboratories) supplemented with 5% ovine blood (CAO). For the inactivation of the culture in TSB, 10% buffered formalin solution was added, in order to obtain a final concentration of 3%, at room temperature for 48 h. An aliquot (0.1 mL) of the formalin-treated culture was streaked on CAO in order to confirm the inactivation of the cells. The inactivated culture was centrifuged at 7000 *g* for 30 min, at 10 °C and the cell pellet was re-suspended in TSB to yield a final concentration of 2.0×10^8 colony-forming unit (CFU) mL⁻¹. This CFU mL⁻¹ was previously standardized using a spectrophotometer (Cintra 5, GBC Scientific Equipment TPTY, Dandenong, Vic., Australia) with a wavelength of 540 nm and colony counts after culture on TSA (Evans *et al.* 2004).

Experiment challenge

Streptococcus agalactiae vaccine master seed was kept in liquid nitrogen in 1.0 mL aliquots until used. The challenge was fermented in 100 mL of TSB at 30 °C for 24 h from a thawed aliquot of the master seed and adjusted to a cell concentration of 3.0×10^7 CFU mL⁻¹ as above. The purity of the challenge was verified by culturing on CAO at 30 °C for 48 h. Pure colonies of *S. agalactiae* were observed.

Treatments

The experiment consisted of two treatments and one control with three replicates (Table 1). In treatment 1, 149 tilapia were intraperitoneally (i.p.) vaccinated with 0.1 mL of vaccine (2.0×10^8 CFU mL⁻¹) and i.p. challenged after 30 days with 3.0×10^6 CFU fish⁻¹. In treatment 2, 133 tilapia were i.p. inoculated with two doses of 0.1 mL vaccine (2.0×10^8 CFU mL⁻¹), at an interval of 21 days and i.p. challenged 30 days after the booster dose with 3.0×10^6 CFU fish⁻¹. In the control group, 130 tilapia were i.p. injected with 0.1 mL sterile TSB and i.p. challenged 30 days later with 3.0×10^6 CFU fish⁻¹. The challenges were performed using the homologous isolate of *S. agalactiae*. Fish were monitored daily over a period of 16 days,

Table 1 Fish per fibreglass container in each treatment and replicates used to evaluate the efficacy of experimentally inactivated *Streptococcus agalactiae* vaccine in Nile tilapia (*Oreochromis niloticus*) reared in Brazil

Treatments	Replicates	Fish/container
1	1	50
	2	50
	3	49
Total	3	149
2	1	49
	2	41
	3	43
Total	3	133
Control	1	44
	2	43
	3	43
Total	3	130

and the disease signs, together with the mortality, were recorded. Dead and dying fish were collected twice a day. During and at the end of the experiment, 23 fish were necropsied, 13 belonging to the control group during the experiment and three at the end of it, one from treatment 1 during the experiment and three from each treatment at the end of the experiment. Blood, cranial kidney, brain, heart, eye, liver and ascitic samples were collected for bacteriological examinations.

Bacteriological examination

Samples from blood, visceral fluid and organs of moribund fish were streaked on CAO plates that were incubated at 30 °C under aerophilic conditions for 48 h. Non-haemolytic colonies with *S. agalactiae* characteristics were Gram-stained and identified by biochemical assays (Holt 1994). In order to obtain a more precise phenotypical and serological characterization, isolated strains were analysed in API 20 Strep Microtest (BioMerieux, Marcy-l'Etoile, France), and classification in the Lancefield group was performed using the Slidex Strepto-kit (BioMerieux), following the recommendations made by Evans *et al.* (2002).

Statistics

The χ^2 -test corrected by Yates was used, with a significance level of 5%. The calculation of the relative risk (RR) with a thrust interval of 95% was performed in order to verify the presence of an association between the vaccine and the protective factor

(Thursfield 2005). EPI6 6.04 (Dean, Dean, Coulombier, Brendel, Smith, Burton, Dicker, Sullivan, Fagan & Arner 1994) statistical package was used. Vaccine efficacy was calculated as the relative per cent of survival (RPS), according to Amend 1981, as the formula

$$\text{RPS} = 1 - \frac{\% \text{mortality of vaccinated animals}}{\% \text{mortality of control animals}} \times 100$$

Results

The results of the efficacy of *S. agalactiae* vaccine in tilapia immunized in treatment 1 were RR 0.16 (0.10–0.27), RPS 83.6% ($P = 0.0001$). In those of treatment 2, RR was 0.03 (0.01–0.11); RPS was 96.6% ($P = 0.0001$). The mortality coefficient for treatment 1 was 10.74% [confidence interval (CI) 95% = 6.49–16.50], and for treatment 2, it was 2.26% (CI 95% = 0.58–6.01). A statistically significant difference ($P = 0.0045$) between the proportions could be observed in both treatments. The control treatment showed a mortality of 64.5%. The mean mortalities for the replicate trials were as follows: 5.3 ± 2.9 in treatment 1, 1.0 ± 1.0 in treatment 2 and 28 ± 6.5 for control group.

Figure 1 shows the daily mortality in the two treatments and the control group after challenge. In treatment 1, 16 fish died (16/149), 13 on the second day after challenge and the remaining three on the 11th

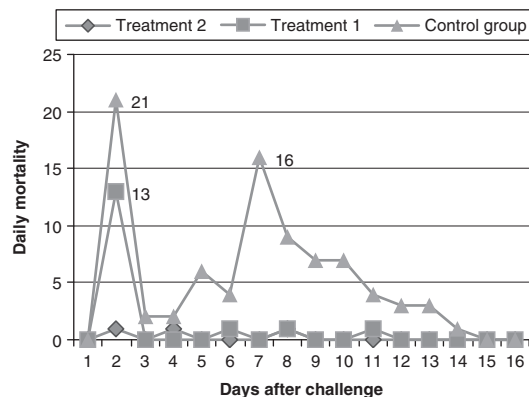


Figure 1 Daily mortality of Nile tilapia (*Oreochromis niloticus*) intraperitoneally vaccinated with one (treatment 1) and two doses (treatment 2) of inactivated *Streptococcus agalactiae* vaccine and challenged after 30 days. Daily mortality of Nile tilapia (*Oreochromis niloticus*) intraperitoneally injected with sterile tryptic soy broth, challenged 30 days later (control group).

day. In treatment 2, three tilapia died (3/133) between the second and the eighth day. In the control group, 85 fish died (85/130), with two well-defined mortality peaks: one on the second day and the other on the seventh and eighth day after the challenge. Tilapia from the control group that died in the first peak presented anorexia, an alteration in skin colour and lethargy. From the fifth day onwards, aggravation of clinical signs could be noticed, with fish presenting anorexia, ascites, erratic swimming, white stains on the body and uni- and bi-lateral exophthalmia.

In fish autopsied from the control group during and at the end of the experiment, macroscopic alterations could be observed, such as yellow, white or bloody liquid present in the visceral cavity, adherence of the organs, an empty stomach, a full gallbladder and a soft and haemorrhagic brain and liver. All fish from treatments 1 and 2 were active and fed normally during the experiment. Tilapia that died in these treatments did not present disease signs, except for one fish from treatment 1, which presented bilateral exophthalmia, lethargy and anorexia on the 11th day after challenged. In these treatments, animals ate viscera and eyes from the dead animals. At the end of the experiment, fish that were necropsied did not present macroscopic alterations, and in the 12 samples of biological material collected from six fish at the end of the experiment, *S. agalactiae* was not isolated. In the 71 samples collected from the brain, visceral liquid, liver, cranial kidney and eye from fish in the control group during and at the end of the experiment, and also in the three samples from one fish in treatment 1 subjected to a bacteriological examination, *S. agalactiae* was isolated, presenting the same morphological, biochemical and serological characteristics of the strain used for the vaccine and challenge. Streptococcal samples were conformed to the Lancifield group B, and the biochemical profile in API 20 Strep Microtest (BioMerieux) was of *S. agalactiae*.

Discussion

The results of the present study showed that an inactivated *S. agalactiae* vaccine produced from a master seed obtained from a disease tilapia in Brazil protected tilapia against the homologous *S. agalactiae* isolate under experimental conditions. Eldar *et al.* (1995) produced an inactivated and protein extract *S. difficile* vaccines. They reported that both inactivated and the protein extract vaccines were protective in tilapia. These vaccines

were produced from master seeds of *S. difficile* isolated from diseased tilapia raised in Israel and were also used in the experimental challenges.

Evans *et al.* (2004) reported that an *S. agalactiae* vaccine i.p. administered to 30 g tilapia provided protection at 26 and 32 °C water temperatures. The vaccine was not protective to 5 g tilapia at either water temperature. Tilapia of 5 or 30 g was only partly protected, when the vaccine was given by bath immersion at either water temperatures. Further, their results showed that an *S. iniae* vaccine did not provide protection against *S. agalactiae*. The master seed and challenge isolates were obtained from a diseased mullet collected in the Kuwait Bay during an epizootic outbreak.

Analysing different experiments with streptococcus vaccines for fish, it could be observed that several factors can influence the efficacy of the vaccine: antigenicity of the streptococcus master seed, composition of the vaccine, concentration of the vaccine, route of inoculation, age of fish, temperature of water, concentration of challenge inoculums, fish species, use of adjuvant and booster dose and other factors. The antigenicity of the vaccine master seed may be the most important factor in the success of a vaccine against heterogeneous isolates of *S. agalactiae*. However, little is known about the antigenicity composition of widely distributed isolates of *S. agalactiae*.

Vaccine concentrations studied by Eldar *et al.* (1995), Evans *et al.* (2004) and by Pasnik, Evans, Panangala, Klesius, Shelby and Shoemaker (2005) varied from 4.0×10^9 to 1.0×10^{10} CFU mL⁻¹, and the concentrations of inoculums used for the challenge of vaccinated and non-vaccinated fish ranged from 2.6×10^3 to 1.7×10^6 CFU fish⁻¹. The variation in the mortality of non-vaccinated fish was from 45% to 100%. In the present study, a vaccine with a concentration of 2.0×10^8 CFU mL⁻¹ and an i.p. challenge of 3.0×10^6 CFU fish⁻¹ was successfully used.

There are few reports of experiments using two doses of streptococcal vaccines. Eldar *et al.* (1995) observed that 100% fish vaccinated with two doses survived the challenge with *S. difficile*. In the present work, tilapia inoculated with two doses of vaccine presented a survival rate of 96.6% and those inoculated with one dose presented a survival rate of 83.6%. Despite the significantly better efficacy of vaccination with two doses, further and more detailed studies must be performed in order to evaluate the cost–benefit.

The results of this study indicated that RPS of 83.6 and 96.4, for a single and booster immunization, respectively, was achieved in Nile tilapia.

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